THE EFFECT OF COLCHICINE ON RECONSTITUTIONAL DEVELOPMENT IN DUGESIA DOROTOCEPHALA[1]

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Many investigators have used the regeneration phenomenon observed in planarians in an attempt to understand the basic mechanisms which control developmental processes. Most of these studies have been made at the organismic level. However, as early as 1904, Bardeen and Baetjer demonstrated that the regenerative powers of *Planaria maculata* and *P. lugubris* could be destroyed by exposing animals to the action of x-rays before section. When pieces were exposed to the radiations only a small blastema developed. Histological observations showed an absence of mitosis in all irradiated pieces while controls showed parenchymal cells in division. Because of the differentiation of imperfect eyes in one case following treatment of the entire animal, these authors suggested that x-rays affected growth processes more than differentiation. Later work with x-rays (Curtis, 1928) and radium (Wiegand, 1930) confirmed the work of Bardeen and Baetjer and showed that large doses selectively destroyed parenchymal cells.

Wolff and Dubois (1948) demonstrated cellular migration in regeneration by grafting a piece of normal tissue into the irradiated anterior 2/5 of the *Euplanaria lugubris*. Donor pigment and cells were found to have migrated through the irradiated host tissues and formed a blastema. The duration of migration varied directly with the distance covered to the regenerating surface.

Curtis and Schulze (1934) made direct counts of the parenchymal cells in species capable of regeneration (*Euplanaria agilis, P. maculata*) and in others possessing a limited capacity to regenerate (*Procotyla fluviatilis*). The only cells observed to be in mitosis in this study were the free parenchymal amoebocytes. Their observations indicated that the capacity to reconstitute is correlated with the number of these cells which they suggest are present as a persistent embryonic stock. Hyman (1951), however, holds that this does not explain the capacity of a post-pharyngeal piece of *P. fluviatilis* to regenerate a tail and not a head.

Colchicine has been used to study cellular changes in limb regeneration in larval urodeles. Thornton (1943) treated larvae of *Amblystoma punctatum* and *A. opacum* with colchicine solutions and found that limb stumps of larvae kept in 1 : 1,500 continuously, from the time of amputation, completely failed to regenerate. While the mechanism of colchicine inhibition of regenerating limbs was not investi-

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gated in this work the author suggested that it was no doubt due to the inhibition of mitosis.

Since work on planarian regeneration has shown that cells migrate from the general parenchyma to the site of injury and contribute to the restoration of the lost parts by division, it appeared that colchicine might be useful in the study of planarian reconstitution. Because colchicine effectively inhibits cells in division (and possibly in migration) in other organisms, the present work was undertaken in an attempt to answer, at least in part, some of the following questions concerned with colchicine effects on planarian reconstitution: (1) Does colchicine inhibit regeneration of planarian pieces? (2) If so, how does this affect the head frequency gradient found in normal regeneration? (3) Is the process of fission altered by colchicine? (4) Are the effects of colchicine treatment reversible? (5) What effect, if any, does colchicine have on physiological dominance in reconstituting pieces of planarians? (6) How does colchicine affect differentiation?

Materials and Methods

The material used in this investigation was the flatworm, *Dugesia dorotocephala*. The stocks were taken from spring-fed streams leading into the Fox River at Cary, Ill. and the Des Plaines River at Schiller Park, Ill. During the course of this work large stocks were successfully maintained in the laboratory. Tap water, that had been aerated for at least 24 hours, was used exclusively. Beef liver was fed approximately twice a week. Only animals that had become adjusted to laboratory conditions and were starved for 7–10 days were used in the final experiments. Unless otherwise stated the animals were 14–16 mm. in length in all experiments. Control and experimental animals were always taken from the same stock.

Studies were made on pieces of various lengths. After sectioning in water, groups of similar pieces were placed in aerated tap water (control) and into various test solutions (prepared with tap water) of colchicine. During the fall, winter and spring the animals were allowed to reconstitute at room temperatures (21–23° C.). During the summer the bowls containing the control and experimental pieces were kept on the water table where fluctuations in temperature were not excessive (17–20° C.).

Preliminary tests showed that solutions of $M/1{,}000$ to $M/4{,}000$ colchicine were excessively toxic and that pieces or entire animals exposed to these concentrations cytolyzed within a few days. Similar tests also showed that $M/15{,}000$ was at the lower limit of concentrations yielding colchicine effects. Thus, concentrations ranging from $M/5{,}000$ to $M/15{,}000$ were used in all experiments reported in this work.

Experimental

1. *Effects of colchicine on regeneration in transverse pieces from different body levels*

Pieces, approximately 1/8th the length of 14–16 mm. animals, taken from different body levels were placed directly into $M/5{,}000$ and $M/10{,}000$ colchicine

for periods of 2, 5, 7 and 10 days after which they were returned to water. Daily observations were made until the 10th day. This series included 250 animals (1250 pieces).

Control pieces differed in degree of head regeneration at various levels. Anterior (A) pieces developed almost 100 per cent normal heads while pieces from more posterior levels showed various degrees of head inhibition. At the most

TABLE I

Designation of regulation values

Type	Assigned regulation value	Control
I	100	Old tissue shows regulation to typical form and proportion of new individual. Head types vary according to level of origin of piece.
		Experimental—Colchicine treatment
II	85	Old tissue shows slightly less than normal regulation to new form and proportion; normal head and tail develop.
III	70	Old tissue shows little regulation to new form and proportion; normal or near normal head and tail develop.
IV	55	Old tissue shows no apparent regulation to new form and proportion; small anterior blastema with eyes develops and a posterior blastema may or may not develop.
V	40	Old tissue shows no apparent regulation to new form and proportion; small anterior blastema without eyes develops and a posterior blastema may or may not develop.
VI	25	Old tissue shows no regulation to new form and proportion; no blastema develops but eyes appear in the anterior cut surface.
VII	10	Old tissue shows no regulation to new form and proportion; pieces remain unchanged since time of cut.

posterior levels (E) almost all were headless.[3] Although many of the control pieces showed head inhibition almost all showed regulation in general body form. That is, the regenerating piece decreased in width and extended in length when compared to the original section cut from the parent.

[3] Head inhibition in short transverse pieces has been described many times (Child, 1911, 1912, 1914, 1920; Child and Watanabe, 1935; Watanabe, 1935; Rulon, 1936; Watanabe, 1941). Such inhibition appears to be the result of two factors, namely, the level from which the piece was taken and the intensity of factors originating from the posterior cut surface (Buchanan, 1922).

It was immediately noted that pieces treated with colchicine could not be compared with the controls on the basis of head inhibition since extended treatment, with the higher concentrations, resulted in little or no new tissue. When such pieces were removed to water those surviving regulated (after 2–3 weeks)

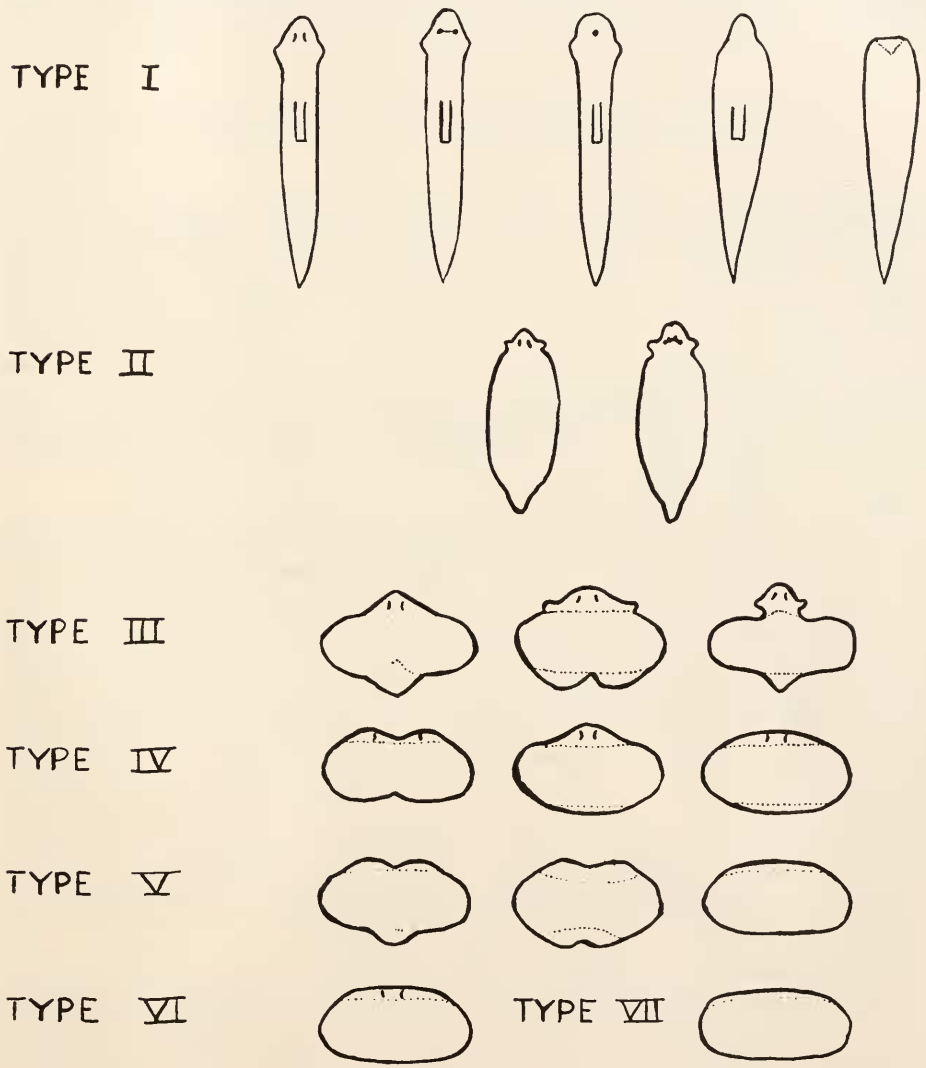

FIGURE 1. Classification of types of regenerates obtained subsequent to colchicine treatment.

to normal body form and regenerated almost 100 per cent typical heads with normal or supernumerary eyes. Since the present experiments were based on observations made 10 days after section, the pieces were classified according to the degree of regulation at that time (Table I, Fig. 1). All regenerates were

classified as to one of 7 types. Type I was the control and was classified as 100 per cent regulated. Types II to VII included those ranging from slightly less than normal regulation to a total lack of change from the time of section. In order to compare data from different experiments (levels, concentrations, etc.) a *Regulation Index* was devised by assigning each type a numerical value which roughly indicated the degree of regulation. Through the use of the assigned value and the number of forms of each type the Index was calculated as follows:

$$\frac{100n + 85n + 70n + 55n + 40n + 25n + 10n}{\text{number of surviving pieces}} = \text{R I (Regulation Index)}$$

One-eighth pieces in *M*/5,000 colchicine for 10 days developed no blastemae and retained the shape and size they had at the time of section (Type VI–VII). All pieces surviving from A and B levels developed eyes buried below the overlying epithelium (Type VI), while at C, D and E levels high percentages remained unchanged (Type VII). After 10 days in *M*/10,000 colchicine all levels showed greater regulation than in *M*/5,000. All surviving A, B and C pieces had small anterior blastemae with eyes (Type IV), while D and E showed a sizable percentage (40 and 36) with small blastemae lacking eyes (Type V). These data then indicate that A and B levels have a greater capacity for eye differentiation than have more posterior pieces. The regulation indices roughly indicate the inhibition effected by colchicine at the different levels.

With two days initial exposure to *M*/5,000 colchicine, regulation was high (by the 10th day) with the indices being above 82 at all levels. With *M*/10,000, the indices were above 94 at all levels. With 5 days exposure to *M*/5,000 there was no regulation of form at any level. However, the anterior pieces showed 100 per cent differentiation of eyes (Type IV), while many posterior pieces failed to show such development. Regulation was greater in all pieces treated with *M*/10,000 but best at posterior levels (D and E). With a 7-day exposure to *M*/5,000 anterior pieces showed the same results as a 5-day exposure but posterior pieces showed a greater capacity to regulate. With *M*/10,000, regulation at 7 days was little different from that of 5 days with the exception of the B level where it was considerably less. All of these data, as well as those for continuous exposure, show that *M*/5,000 colchicine is more inhibitory at all exposure periods than is *M*/10,000. These data also show that anterior pieces (A and B) exposed for 5, 7 and 10 day periods to *M*/5,000, while lacking the capacity for regulation of form and growth, give 100 per cent differentiated eye spots.

The consistent appearance of eye spots at anterior, but not at posterior levels, is in conformity with the gradient work of Child (1911). It must be pointed out, however, that these are not in differentiated heads but in completely unregulated pieces without blastemae. With the exception of a 5-day initial and a 10-day continuous exposure to the high concentrations, posterior pieces showed greater regulation and less susceptibility to the toxic effects of colchicine than did anterior pieces.

In general, these experiments have shown that posterior pieces have greater capacity for blastema formation and viability while anterior pieces have a greater capacity for differentiation of eye spots.

2. A critical period in reconstitution as determined by the use of colchicine

Since the data on transverse pieces, in high concentrations of colchicine, showed that 5 days' exposure was more lethal and permitted less regulation than 7 days (pieces D and E in *M*/5,000 colchicine) a critical period in development, centering around 5 days, was indicated. In order to identify this period more accurately

TABLE II

Comparison of Regulation Indices of 1/6th pieces when treated with M/5,000 and M/7,000 colchicine for 1–9 days after section. (Data in per cent.)

Level	1 day		2 days		3 days		4 days		5 days	
	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index
A	0	100								
B	0	100								
Control C	0	100								
D	0	100								
A	0	91.1	0	97	48	82.6	38	48.5	30	55.6
B	0	94.6	2	78	62	85	72	35.6	62	43.9
M/5,000 C	16	98.9	0	90.4	48	83.2	40	36.5	60	70
D	0	100	0	94.6	18	75.3	11	22.3	22	63.2
A	0	100	0	100	0	100	0	53.2	0	100
B	0	100	0	98.8	44	95.9	0	52.6	32	79.8
M/7,000 C	0	100	0	98.8	20	100	0	40	0	94
D	0	100	0	100	0	91.6	0	55	0	96.8

Level	6 days		7 days		8 days		9 days			
	Dead	Index	Dead	Index	Dead	Index	Dead	Index		
A										
B										
Control C										
D										
A	40	17.5	80	25	80	15	100	10		
B	100	16.5	98	25	100	11.1	100	10		
M/5,000 C	80	25.6	96	13	80	10.6	100	13		
D	46	64.7	96	20	100	13	100	13		
A	64	65	84	12.4	72	14.2	96	25		
B	84	55.5	92	25	92	25	96	10		
M/7,000 C	60	59.5	96	16	92	12.3	100			
D	56	90	60	37.8	92	10	96	25		

(if such actually existed) a further study was made in which slightly larger pieces were exposed to colchicine for more closely graded intervals.

In these experiments, pieces approximately 1/6th the post-cephalic length were used. These pieces were exposed to *M*/5,000 and *M*/7,000 colchicine for 1, 2, 3, . . . 9 days. At the end of each period the pieces were returned to water.

Records were made on the tenth day. An additional analysis was undertaken by treating the pieces with *M*/5,000 colchicine beginning at different time intervals after section. The data, showing treatment with colchicine immediately after cutting, are shown in Table II while those showing treatment at delayed intervals are shown in Table III.

Because so many data are involved (1,075 animals or 4,300 pieces) only Regulation Indices and viability for the different levels are given. It is shown in Table II that exposure for 4 days (not 5, as suggested in the previous section) is more effective in inhibiting growth, and regulation, in both *M*/5,000 and *M*/7,000, than either 3 or 5 days. This difference, relative to exposure time, could not be found in viability (as shown for 1/8th D and E pieces) possibly because the pieces in the present experiment were slightly larger.

The critical period for regeneration and regulation was not shown when pieces were placed into *M*/5,000 colchicine on the 4th day after section (Table III).

TABLE III

Regulation Indices of 1/6th pieces when kept in water for 1–6 days after section and placed into M/5,000 colchicine for the remainder of a 10-day reconstitutional period. (*Data in per cent.*)

Level	1 day		2 days		3 days		4 days		5 days		6 days	
	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index
A	0	100										
B	0	100										
Control C	0	100										
D	0	100										
A	2	54.6	2	54.3	32	54.5	40	56.3	28	62.5	42	64.3
B	26	52.5	42	55	44	53.3	56	60.5	42	64.6	38	55
M/5,000 C	8	48.7	26	47.2	48	48	56	55.7	22	51.1	22	68
D	0	49.3	0	57.2	30	51.5	34	56.3	14	74.8	30	70.4

However, these data show that 4-day regenerates from all levels are more sensitive to the lethal effects of colchicine than are pieces which have regenerated for 3 or 5 days.

Sensitivity of developmental processes to inhibiting agents has been shown to be associated with cleavage and differentiation. Rulon (1950) demonstrated that the fertilized eggs of *Dendraster* were more sensitive to thiourea treatment than were blastulae. Blastulae tolerated high concentrations that were lethal to newly fertilized eggs. Changes associated with reconstitution appear to include an initial adjustment to the effect of section and perhaps dedifferentiation, followed by reorganization including cell proliferation with the final return to species character. It is suggested that the critical period noted here, with its counterpart in normal development, is associated with that time in development when reorganization and cell proliferation are occurring in the reconstitutional process. Histological studies, to be reported later, should verify this proposal.

3. The effect of colchicine on regeneration when whole animlas are treated prior to section

The preceding work showed that planarian pieces were inhibited in both reconstitution and regulation of form when exposed to certain concentrations of colchicine. Obviously the colchicine was exerting its effects on tissue which had been stimulated to regenerate by cutting. The problem which then presented itself dealt with the effect of colchicine on unactivated tissue. To test this, uninjured entire animals were exposed to various concentrations of the agent for 2–6 days after which they were sectioned into 1/6th pieces and permitted to reconstitute in water. Because these experiments were performed in summer, all intact animals and pieces were kept on the water table at 17–19° C. The data were taken on the tenth day following section. This experiment included 200 animals. In general, pieces from treated animals behave much the same way as if they, themselves, had been exposed to the agent. That is, there was prolonged inhibition of regulation and blastema formation in the higher concentrations. The most

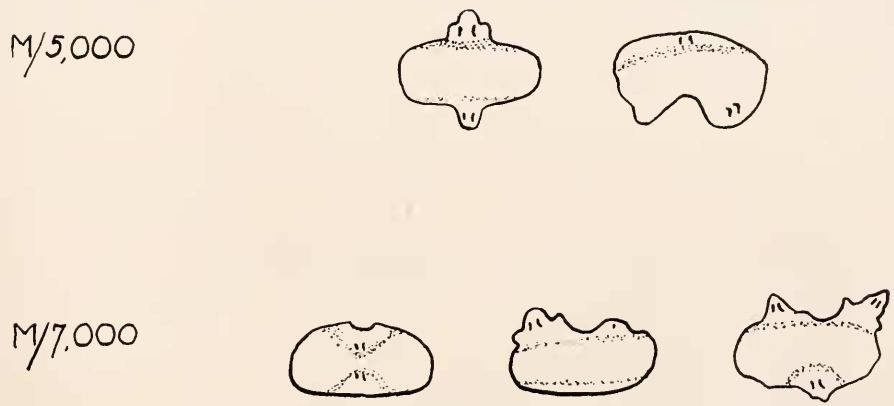

FIGURE 2. Types of heteromorphic forms obtained from 1/6th pieces of 14–16 mm. animals pre-treated with colchicine different lengths of time (*M*/5,000 for 4 days; *M*/7,000 for 6 days). Whole animals and pieces maintained at 17–19° C.

important difference between the regenerates obtained here and those from treatment following section occurred in the development of a high percentage (36 per cent at D level) of heteromorphic forms when the whole animals were treated for 4 days with *M*/5,000 and 6 days with *M*/7,000 colchicine. Regenerates classified as heteromorphic included: (1) simple bipolars, (2) unregulated bipolars (with or without blastemae), (3) poorly regulated forms with two heads at the anterior end, and (4) poorly regulated forms which have regenerated two heads anteriorly and one posteriorly (Fig. 2).

A slightly different series was prepared in which the intact animals were kept at room temperature (21–23° C.) in *M*/2,000, *M*/5,000 and *M*/10,000 colchicine for only two days. After this exposure the animals were cut into 1/8th instead of 1/6th and permitted to reconstitute in water, also at room temperature. This experiment included 225 animals.

This second series showed that increased temperature, with a reduction in

exposure time and size of piece, resulted in considerably less regulation when whole animals were treated with $M/5{,}000$ prior to section. When treated with $M/10{,}000$, under these changed conditions, regulation was also decreased, but it was noted that approximately 50 per cent of those pieces classified as Type V (small blastemae, no eyes) had two separate blastemae at each end of the piece.

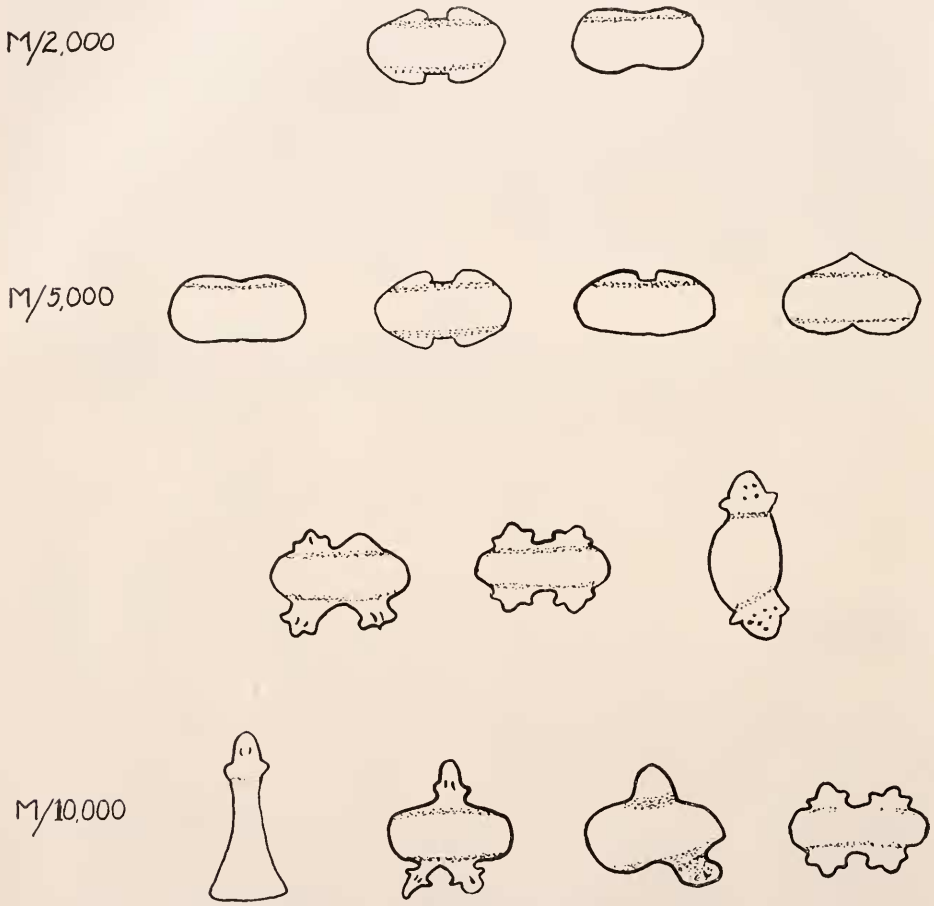

FIGURE 3. Types of regenerates obtained from 1/8th pieces of 14–16 mm. animals pre-treated with colchicine for two days. Whole animals and pieces maintained at 21–23° C.

High percentages (28–44) of heteromorphic forms also appeared. In general, these were similar to those found in the first series but with the addition of a 5th type which had regenerated two heads at each end of the piece (Fig. 3). At D level more than 50 per cent of these forms had 3 or 4 heads.

DISCUSSION AND CONCLUSIONS

This work has shown that certain concentrations of colchicine markedly inhibit regeneration and regulation to normal body form in planarians. It has shown that the degree of inhibition was roughly proportional to the concentration

and that the character of the regenerate showed a high degree of consistency. In all experiments using solutions below the toxic limits it was observed that pieces which survived early treatment with colchicine developed normal heads and attained normal body form and proportion by 10–15 days after return to water. The head frequency gradient (Child, 1913, 1914; Buchanan, 1922; Rustia, 1925; Rulon, 1936, 1938; Watanabe, 1935, 1941) was, therefore, obliterated by this agent. In tap water, short transverse pieces from D and E levels regenerate largely into headless, or near headless forms. It has been found (Buchanan, 1922; Rulon, 1948 and others) that factors inhibiting normal head regeneration arise at the site of posterior section and that these factors may be blocked by nerve anaesthetics and other agents. Such head-inhibiting factors are of short duration since a 24-hour delay in making a posterior cut will permit the development of 100 per cent normal heads (Child and Watanabe, 1935). The present experiments indicate that since colchicine prevents regenerative changes in the piece over an extended period, the head-inhibiting posterior effects have subsided by the time the pieces are returned to water.

The gradient of eye-spot formation, however, was still apparent after 10 days since exposure to high concentrations for this period of time resulted in 100 per cent eye-spots in pieces from anterior levels while no more than 56 per cent of the posterior pieces showed such development. Even though growth and regulation of these pieces was totally inhibited, differentiation was expressed in the appearance of eyes in pieces otherwise unchanged since the time of isolation. This indicates that while colchicine inhibits regeneration it does not at the same time totally inhibit differentiation.

Many investigations have shown a correlation between the presence of nerve tissue and the capacity to reconstitute to normal. Olmsted (1922) has shown that certain polyclads can restore missing parts only if cephalic ganglia are left intact. Silber and Hamburger (1939) and Beyer and Child (1930) observed head development on the medial surface of lateral pieces and suggested that the nervous system plays a role in the localization of head regeneration through its influence on the condition of the cells near it. In the present experiments it is possible that the appearance of eyes below the epithelium at the anterior cut surface of a piece is also correlated with the localizing influence of the nerve cords.

The occurrence of regenerates possessing 3–4 blastemae or 3–4 heads may find its explanation in the obliteration of the medio-lateral differential or gradient. All head formation in normal reconstitution is initiated at the medial point of a cut surface and progresses laterally. The occurrence of two blastemae or two heads at a cut surface (Figs. 2, 3) indicates that the medio-lateral gradient is reduced. A high percentage of pieces taken from animals treated with colchicine before section showed the formation of paired blastemae or heads at both cut surfaces. Apparently colchicine effectively destroys the medio-lateral differential and in so doing frees each side of the piece for independent differentiation. The localizing influence of the nerve cords may be operative here also, as it appears to be in eye spot differentiation after treatment with colchicine.

Summary

1. Colchicine was shown to inhibit regeneration in pieces of *Dugesia dorotocephala*. In addition it was shown to inhibit regulation to normal form.

2. Within certain limits of concentration, the inhibitory effects of colchicine were reversible. Two to three weeks after return to water pieces from all levels developed normal heads (some with supernumerary eyes). Thus, through the use of colchicine it was possible to obliterate the head frequency gradient characteristic of this species.

3. While colchicine was found to exert a marked inhibition of blastema formation in the reconstitution of planarian pieces it did not have the same effect on eye-spot differentiation. Pieces treated with higher concentrations of the agent failed to show any new tissue at the anterior and posterior cut surfaces by 10 days after section. Eye-spots, however, differentiated beneath the covering epithelium in anterior pieces.

4. Heteromorphic forms with 3–4 heads developed from pieces taken from whole animals exposed to colchicine. This indicates that the medio-lateral differential was reduced subsequent to colchicine treatment and that the two lateral halves of the piece underwent independent differentiation.

5. With the use of colchicine evidence was obtained that the 4th day of reconstitution represents a critical period in development. That is, pieces treated with $M/5{,}000$ colchicine for 4 days, and then returned to water, showed more inhibition than pieces treated for 3 or 5 days.

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